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(54) Title: REGULATORY CASSETTE FOR EXPRESSION VECTORS

(57) Abstract

Recombinant DNA, e.g. in the form of a plasmid, comprising a cassette as derived from a *Pseudomonas* TOL plasmid, the cassette comprising a combination of the *xyl*R gene and an operator promoter region stimulated by the *xyl*R gene product. The cassette can be used to regulate gene inserts, to give expression under the control of widely-available aromatic compounds such as toluene.

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REGULATORY CASSETTE FOR EXPRESSION VECTORS

Field of the Invention

This invention relates to a regulatory cassette (or unit) which can be used to construct expression vectors which can be transferred into and maintained in a variety of microorganisms. Such expression vectors, under the control of the cassette, enable the microorganisms to produce desirable biological products in an efficient manner.

Background of the Invention

Bacterial expression systems which are controlled by the insertion of regulatory genes are known. For example, there are systems which are designed for the regulation of E. coli genes. However, there are few expression vectors for gram-negative, non-enteric bacteria. An example of such an organism is Xanthomonas which produces the extracellular polysaccharide xanthan used in microbiologically-enhanced tertiary oil recovery. Xanthan gum also has widespread use as a food additive and as a lubricating agent. Other examples of organisms which secrete products of commercial importance are Acinetobacter which produces polysaccharides which can be used for cleaning oil spills, Azctobacter which synthesises compounds of use as food additives, Erwinia which secretes pectins of use for clarifying fruit juices, and Pseudomonas which has a wide range of metabolic activities which can be employed in bio-transformation processes.

There are few expression vectors which are regulated by cheap, readily-available compounds such as toluene or benzyl alcohol. Such compounds act via regulatory genes such as xylR (present in TOL plasmids). The nucleotide sequence of the xylR promoter region in the archetypal

TOL plasmid pWWO is given by S. Inouye et al, J. Bacteriol. 163, 863-869 (1985); see Fig. 5.

The regulatory genes have been isolated from the TOL plasmid pWW53 from Pseudomonas putida. The TOL plasmid codes for the degradation of toluenes and xylenes (H. Keil et al, J. Bacteriol. 164, 887-885 (1985), and H. Keil et al, J. Bacteriol. 169, 764-770 (1987)). In the presence of toluene or xylene, the regulatory protein XylR stimulates expression from the operator promoter region p_u, allowing the transcription of the corresponding polycistronic mRNA.

The TOL plasmid pWW53 is distinct from other known TOL plasmids, e.g. pWW0, as is evident from the restriction maps given by H. Keil et al, J. Gen. Microbiol. 133, 1149-1158 (1987). Fig. 1 of that reference gives the restriction map of the complete 36 kbp insert of pWW53 DNA in the cointegrate plasmid pWW53-4. The mapping is discussed only in terms of its evolutionary interest.

Summary of the Invention

It has now been realised that the juxtaposition of the regulatory gene xylR and its binding site (they are only about 1.5 kb apart) in pWW53 is of considerable commercial potential.

A regulatory cassette according to the present invention comprises a combination of substantially all of the regulatory gene xylR, the binding site of its gene product XylR, and the associated promoter (p_u), e.g. as is present in the TOL plasmid pWW53 found in strain Pseudomonas putida MT53. The cassette is introduced into DNA of the same or a different host, by recombinant technology. An expression vector of the invention, containing genes for which increased product expression is desired, includes the cassette. The xylR/p_u cassette will allow efficient expression of genes which are placed

downstream of p_u, in the presence of simple and cheap aromatic compounds, e.g. having 6-12 carbon atoms, such as toluene or benzyl alcohol.

By virtue of the present invention, genes coding for certain functions of gram-negative bacteria can be expressed in a controlled manner in the same host as that from which they were originally cloned. As a result, the cloned genes are well expressed and are unlikely to produce products which are detrimental to the host bacteria's physiology. Alternatively, the cassette can also be used for heterologous gene expression in gram-negative bacteria.

Description of the Invention

The expression vectors of the present invention may be based on known plasmids such as RSF 1010, into which a regulatory cassette based on xylR/p_u is inserted. All DNA sequences necessary for the construction of plasmids (to carry the regulatory cassette) which exemplify the invention have been physically mapped and analysed.

An illustrative expression vector of the present invention comprises a cassette which has been isolated from the TOL plasmid pWW53. The regulatory cassette, which is generally required to be substantially free of functional DNA other than the xylR gene and p_u, can be isolated in a number of ways, using techniques known to those skilled in the art.

A process for preparing the cassette comprises cleaving a Pseudomonas TOL plasmid (with restriction endonucleases) to obtain a fragment containing most or all of the combination. If necessary or desired, a further step comprises removing any genes and/or undesirable restriction sites from the fragment other than the xylR gene by further gene manipulations. For example, deletion may comprise one or more DNA sequences

(of any appropriate length) including a BstEII site within the non-coding region between xylR and the operator promoter region. The product comprises the promoter for xylR and its ribosome binding site, and the promoter for p_u and its ribosome binding site. Convenient restriction sites flanking the combination are available for conventional manipulation, without affecting the desired function of the combination.

The cassette can then be used to construct an expression vector. The basic replicon should be capable of being easily transferred and stably maintained in a range of bacteria, in particular the gram-negative non-enteric bacteria such as Pseudomonas, Xanthomonas and Azotobacter. For example, the cassette can be inserted into a derivative of RSF 1010; other plasmids or their derivatives which have similar characteristics to the parental plasmid may be used to contruct expression vectors of the present invention.

There are three main types of expression vectors which exemplify the present invention. The specific details are given for the purposes of illustration only.

The first type of vector consists of plasmid pKT 230, which is a derivative of RSF 1010, into whose unique BamH1 and EcoRI sites the xylR/p_u cassette is inserted. EcoRI, SacI and KpnI restriction fragments can be inserted downstream of p_u. This expression vector can allow the controlled expression of any gene which is inserted in these restriction sites, in the presence of compounds such as toluene or benzyl alcohol.

The second type of expression vector is a cascade expression vector using the leftward promoter from bacteriophage λ (λp_L) and its thermolabile repressor protein cI857 which is inactive at 42°C, in combination with the same xylR/p_u cassette constructed as described above for the first type of vector. This sequence is

inserted as EcoRI fragment into the EcoRI site of pPLGN1 which already carries λp_L and the cI857 gene. KpnI and SacI restriction fragments can be inserted downstream of p_u. In this vector system, the initial induction is achieved by raising the temperature, say from 28°C to 42°C, leading to the over-expression of XylR from λp_L. This over-expression of XylR in turn leads, in the presence of inducers (e.g. toluene or benzyl alcohol), to a high level of transcription of a gene inserted downstream of p_u. This cascade induction system is particularly useful because, if the native weak xylR promoter is retained, the two-level expression vector can be used to give moderate induction in the presence of the hydrocarbon inducers during exponential growth and, at the end of the growth phase, very high levels by increasing the temperature to 42°C.

The efficiency of both vectors can be monitored by inserting appropriate indicator genes into the insertion sites such as the CDO (catechol 2,3-dioxygenase) or lacZ gene.

The usefulness of vector systems of this second type can be further demonstrated by inserting the genes necessary for the conversion of toluene to benzoate downstream of p_u. The efficient conversion of aromatic hydrocarbons into carboxylic acids is a potentially valuable bio-transformation process.

The third type of vector is a highly repressible expression vector. It is constructed in a similar manner to the second type of vector, but with the transcriptional orientation of the xylR/p_u cassette facing towards λp_L at the pPLGN1 EcoRI site; again SacI and KpnI restriction fragments can be inserted. The transcription of a gene cloned into these sites in either orientation can be highly repressed by inducing the promoter downstream of the insert, thus generating high

levels of anti-sense mRNA, while its own promoter remains switched off. Induction can be achieved simply by repressing the anti-sense mRNA promoter, e.g. by temperature shift, and inducing the gene's own promoter, e.g. by adding toluene to the system.

This third vector type will allow the tight repression of potentially toxic products which, if produced in significant quantities during exponential growth, would be deleterious to the host cell. The efficiency of this vector can be assessed by, for example, inserting genes for restriction endonucleases such as EcoRI into the insertion sites, and analysing the survival of a modification-deficient host strain under appropriate induction/repression conditions.

In general, a host such as a gram-negative bacterium including an expression vector of the invention can be used to produce products encoded by a gene inserted downstream of, and controlled by, the xylR/p_u combination. Not only hydrocarbon to aromatic carboxylic acids-converting enzymes, but also biological polypeptides such as Factor VIII, interleukins, interferons etc. may be produced. Since the inducer is cheap, large vessels may be used first to produce high biomass and then to give expression, on induction. In addition the inducers can be removed relatively easily from the desired end-products (e.g. by distillation).

The following procedures, of which the products of the respective steps are shown schematically in the accompanying drawing, and identifed there by pEHK numbers, illustrate the invention.

Preparation

The initial task was to obtain a recombinant plasmid carrying both xylR and p_u, next to one another, on the same restriction fragment. This was achieved by cloning the 17.3 kb EcoRI fragment from TOL plasmid pWW53-4 into

the cloning vector pUC19 in order to obtain the first plasmid shown in the drawing (described as pEHK11). This plasmid carried several superfluous genes from the TOL pathway in the upstream region and a short interfering DNA segment downstream, all of which should be removed. The TOL genes xyl LEGF were deleted by SacI subcloning of pEHK11, to obtain pEHK13.

pEHK13 was partially restricted with BamH1 and Bg1II and a 5.2 kb region was obtained which carried no functional TOL genes upstream of xylR. This DNA construct (pEHK15) carried a unique BamH1 site downstream of the coding region of xylR and, less than 50bp to its right, a BstEII site (one of three on this insert).

The purpose of succeeding steps was to obtain both xylR and p_u on a BstEII fragment, because there would thus be very convenient border sequences on either side: on the left-hand end, only the promoter and ribosome binding site (PBS) of xylR; on the right-hand end, p_u and its RBS but almost no non-essential and hence potentially deleterious DNA. To this end, pEHK15 DNA, which has three BstEII sites, was taken, restricted at the unique BamH1 site which was known to be located downstream of the xylR gene and only about 50 nucleotides away from the central BstEII site (H. Keil et al., J. Bacteriol. 169, supra) and subsequently treated with exonuclease Bal31 at a rate which allowed the continuous removal of between 0.1 to 1.0 kb in both directions. The DNA was then blunt-ended by PolIk treatment and religated with T4-DNA ligase. After transformation into E. coli ED8654, 48 colonies were analysed for their DNA profile and found to have deletions ranging in length from 0.1 to 1.0 kb. They had all lost their central BstEII site and hence gave rise to a unique BstEII fragment of variable length of between 3.9 and 3.0 kb containing all or part of xylR + p_u (pEHK250 series). DNA from 6 representative clon s

was isolated, one of which (pEHK 255) carried a 3.4 kb BstEII insert and was restricted with BstEII followed by PolIk incubation; this DNA was then inserted into the unique SmaI site of M13 mp19. The bacteriophages from several white plaques were isolated and their DNA was characterised. One construct (pEHK355) carried the original 3.4 kb BstEII insert with unique SacI, KpnI and EcoRI sites downstream of p_u restriction sites, suitable for the insertion of foreign genes.

The construct thus obtained was excised with BamH1/EcoRI and inserted into the broad host range vector pKT230 also cut with BamH1/EcoRI, in order to obtain the expression vector pEHK455. After insertion of, say, the CDO indicator gene into the unique EcoRI site, the constructs could be analysed for their function in E. coli or Pseudomonas, after addition of the inducer toluene, via SDS-PAGE analysis of the protein profile or via quantitative spectroscopic enzyme activity measurements.

In a particular case, the recombinant plasmid pEHK455 was restricted with EcoRI and the CDO gene (about 1.6 kb) was inserted as EcoRI fragment, and the construct subsequently transformed into E. coli. Improved expression was observed upon addition of toluene.

The second and third constructs described above, i.e. cascade expression vectors and highly repressible expression vectors, could be obtained from the above construct in one step, by restriction at the unique BamH1 site, filling in the ends by PolIk, adding EcoRI linkers in the presence of T4-DNA ligase, and inserting the resulting fragment into a EcoRI restricted vector such as pBR325 or directly into pPLGN1; the orientation of insertion determines which type of construct has been obtained.

CLAIMS

1. A regulatory cassette as derived from a Pseudomonas TOL plasmid, which comprises the xylR gene in combination with a promoter stimulated by the xylR gene product.

2. A regulatory cassette according to claim 1, which comprises no genes from the TOL plasmid other than the xylR gene.

3. A regulatory cassette according to claim 1 or claim 2, in which the gene-promoter combination is as found in Pseudomonas putida.

4. A regulatory cassette according to claim 3, in which the gene-promoter combination is as found in plasmid pWW53.

5. DNA, e.g. in the form of a plasmid, comprising a heterologous cassette according to any preceding claim.

6. An expression vector comprising a replicon and a regulatory cassette according to any of claims 1 to 4, the cassette allowing improved expression of a gene insert in the vector.

7. A vector according to claim 6, which contains a promoter additional to and heterologous with the promoter stimulated the xylR gene product.

8. A vector according to claim 7, in which the promoter is from bacteriophage λ.

9. A vector according to any of claims 6 to 8, which additionally comprises a gene for a thermolabile repressor protein.

10. A vector according to any of claims 6 to 9, which additionally comprises a gene transcribing anti-sense mRNA which, if induced, represses the xylR gene and, if repressed, allows expression of the xylR gene.

11. A vector according to any of claims 6 to 10, which has a multi-cloning site downstream from the gene-promoter combination.

12. A vector according to any of claims 6 to 11, which is regulated by toluene, benzyl alcohol or another C_{7-12} aromatic compound.

13. A gram-negative, non-enteric bacterium including an expression vector according to any of claims 6 to 12.

14. A process for converting an aromatic hydrocarbon to an aromatic carboxylic acid, which comprises cultivating a bacterium according to claim 13 in the presence of the aromatic hydrocarbon.

15. A method of producing a regulatory cassette according to any of claims 1 to 4, which comprises the steps of:

a) cleaving the Pseudomonas TOL plasmid to obtain a fragment containing most or all of the gene-promoter combination; and, if necessary,

b) removing any genes from the fragment other than the xylR gene; and/or

c) adding a oligonucleotide to replace any essential missing part of the combination.

16. A method according to claim 15, for producing a cassette according to claim 4, wherein cleaving comprises digestion with a EcoRI or EcoRI* restriction enzyme, and genes upstream from the xylR gene are then uni-directionally deleted from the fragment.

17. A method according to claim 15, for producing a cassette according to claim 4, wherein cleaving comprises digestion with a BglII restriction enzyme, and a 50-80 bp oligonucleotide is then added to the fragment.

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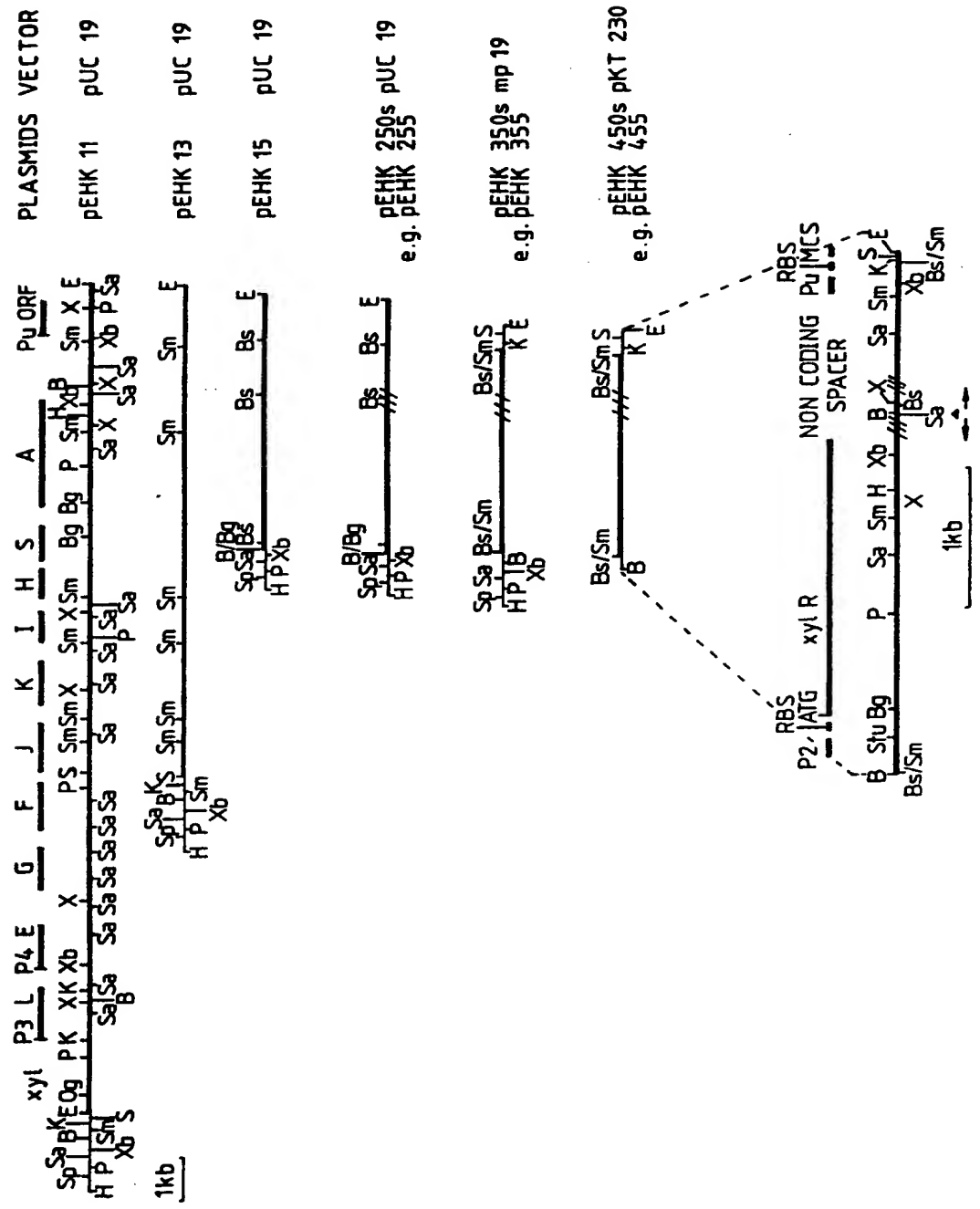
PLASMIDS VECTOR
pEHK 11 pUC 19
pEHK 13 pUC 19
pEHK 15 pUC 19
pEHK 250s pUC 19
e.g. pEHK 255
pEHK 350s mp 19
e.g. pEHK 355
pEHK 450s pKT 230
e.g. pEHK 455
xyl
Pu ORF
RBS
NON CODING SPACER
xyl R
1kb

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 89/00341

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [5]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 N 15/00, C 12 N 1/20, C 12 P 7/40

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Journal of General Microbiology, vol. 133, no. 5, May 1987, SGM, (Colchester, GB), H. Keil et al.: "Molecular analysis of regulatory and structural xyl genes of the TOL plasmid pWW53-4", pages 1149-1158 see figure 1 cited in the application --	1,3,4
X	Chemical Abstracts, vol. 103, no. 21, November 1985, (Columbus, Ohio, US), S. Inouye et al.: "Determination of the transcription initiation site and identification of the proteins product of the regulatory gene xylR for xyl operons on the TOL plasmid", see page 186, abstract 173058y, & J. Bacteriol. 1985, 163(3), 863-9 cited in the application --	1-3

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* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
21st June 1989	27 JUIL. 1989
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	Febs Letters, vol. 226, no. 2, January 1988, Elsevier Science Publishers B.V., (Biomedical Division), (Amsterdam, NL), J.L. Ramos et al.: "Broad-host range expression vectors containing manipulated meta-cleavage pathway regulatory elements of the TOL plasmid", pages 241-246 see the whole article --	1-17
A	Chemical Abstracts, vol. 105, no. 17, 27 October 1986, (Columbus, Ohio, US), N. Mermod et al.: "Vector for regulated expression of cloned genes in a wide range of Gram-negative bacteria", see page 197, abstract 147488k, & J. Bacteriol. 1986, 167(2), 447-54 --	1-17
A	Journal of Bacteriology, vol. 164, no. 2, November 1985, American Society for Microbiology, (Baltimore, US), H. Keil et al.: "Evolutionary conservation of genes coding for meta pathway enzymes within TOL plasmids pWWO and pWW53", pages 887-895 see the whole article cited in the application --	1
A	Proc. Natl. Acad. Sci. USA, vol. 84, no. 15, August 1985, (Washington, DC, US), S. Inouye et al.: "Expression of the regulatory gene xylS on the TOL plasmid is positively controlled by the xylR gene product", pages 5182-5186 see the whole article --	1,3,4-6
A	EP, A, 0242220 (NOVO INDUSTI A/S) 21 October 1987 see the whole document ----------	1-17

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 8900341 SA 28030

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 20/07/89
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0242220	21-10-87	JP-A- 63157987	30-06-88